Theor Appl Genet (1999) 99:1179–1186

C.J. Liu · J.M. Musial · B.D. Thomas

Genetic relationships among *Stylosanthes* species revealed by RFLP and STS analyses

Received: 18 September 1998 / Accepted: 23 March 1999

Abstract Genetic relationships between 6 unclassified taxa and 24 known species of the genus *Stylosanthes* were investigated by RFLP and STS analyses. This allowed the diploid taxa used in this study to be classified into nine basal (genome) groups. Representative species in these groups included 'S. seabrana'/*S. hamata* (Group A), *S. viscosa* (Group B), *S. humilis* (Group C), *S. macrocephala/S. bracteata* (Group D), *S. pilosa* (Group E), *S. leiocarpa* (Group F), *S. guianensis* (Group G), *S. tomentosa* (Group H) and *S. calcicola* (Group I). Polyploid taxa used were grouped into five classes based on their putative genomic structures. These are AABB for *S. scabra*, *S.* aff. *scabra*, *S. sericeiceps*, *S.* aff. *hamata* and *S. tuberculata*; AACC for *S. mexicana*, *S. subsericea*, *S. sundaica* and *S. sp.*A; DDEE for *S. capitata*; AAFF for *S. sympodialis*; and AABBXX for *S. erecta*, with XX representing an unknown genome. Of the 6 unclassified taxa, three were diploids and 3 tetraploids. Of the 3 diploids, the genome of *S. sp.* was markedly distinct from those of all other diploids analysed in this study, with that of *S. leiocarpa* being the closest. The genome of *S. sp.*B was similar to that of *S. humilis*, with an average dissimilarity value of 15% between them. The genome of *S.* aff. *viscosa* was very similar to that of *S. viscosa*. Genetic variation between these 2 taxa was not larger than that within each of the 2 taxa. Of the 3 tetraploids, the genomic structure of *S. sp.*A was similar to those of *S. mexicana, S. sundaica* and *S. subsericea*, and the genomic structures of *S.* aff. *scabra* and *S.* aff. *hamata* were similar to those of *S. scabra* and *S. sericeiceps*.

Communicated by G.E. Hart

C.J. Liu (✉) · B.D. Thomas
CSIRO Tropical Agriculture,
306 Carmody Road,
St Lucia, QLD 4067, Australia
e-mail: Chunji.Liu@tag.csiro.au
Fax: 61 7 3214 2288

J.M. Musial
CRC for Tropical Plant Pathology,
University of Queensland, St Lucia,
QLD 4072, Australia

Key words *Stylosanthes* · DNA markers · Genetic relationships

Introduction

The genus *Stylosanthes* is naturally distributed in tropical, subtropical and warm temperate regions of the Americas, tropical Africa and South-East Asia. Based on morphological classifications, the genus comprises about 40 species and a variable number of subspecies and varieties depending on whose taxonomy is accepted (Williams et al. 1984). They can be diploid, tetraploid or hexaploid, all having a basic chromosome number of x=10 (Cameron 1967). Several of these species are important forage legumes in tropical regions (Edye and Cameron 1984). In northern Australia, allotetraploid *S. scabra* and *S. hamata* form, by far, the most important introduced forage legume (Cameron *et al.* 1996).

Species identification in *Stylosanthes* based on morphological characters is difficult. Different viewpoints exist about species concepts and about what characters are suitable for species identification (Mannetje 1984; Sousa Costa and Ferreira 1984). As a result of these difficulties, there has been disagreement over species status for a large proportion of the genus (Mannetje 1984; Williams et al. 1984). In addition, there are a substantial number of unnamed accessions in *Stylosanthes* collections (Schultze-Kraft et al. 1984).

Molecular markers have proven to be powerful in elucidating genetic relationships in a wide range of species. In *Stylosanthes*, randomly amplified polymorphic DNA (RAPD) has been used to investigate genetic relationships between members of the *S. guianensis* species complex (Kazan et al. 1993a, b) and between *S. scabra* and *S. fruticosa* (Glover et al. 1994); restriction fragment length polymorphisms (RFLP) were used to identify putative diploid progenitors of allotetraploid *S. hamata* (Curtis et al. 1995); sequence-tagged-sites (STS) were used to identify progenitor species for *S. scabra* (Liu and Musial 1997) and chloroplast DNA variation was used to

Table 1 The *Stylosanthes* genotypes used and their origins

CPI	Species	Genotype code	Country	Latitude	Longitude	Ploidy	Genome
92463	*seabrana*	Seal	Brazil	12°20′S	41°52′W	D	A
110361	*seabrana*	Sea2	Brazil	12°33′S	41°06′W	D	A
57247	*hamata*	Ham1	Venezuela	11°11′N	68°49′W	D	A
110107	*hamata*	Ham2	Venezuela	9°38′N	69°47′W	D	A
33831	*viscosa*	Vis1	Mexico	22°25′N	97°55′W	D	B
40300	*viscosa*	Vis2	Brazil	3°46′S	38°34′W	D	B
33941	aff. *viscosa*	Vial1	Mexico	16°11′N	95°12′W	D	B
91317	aff. *viscosa*	Via2	Mexico	16°10′N	95°13′W	D	B
55797	*sp.* B	Spb1	Brazil	9°22′S	40°30′W	D	C
33502A	*humilis*	Hum1	Venezuela	9°47′N	63°33′W	D	C
33979	*humilis*	Hum2	Costa Rica	10°26′N	85°08′W	D	C
78478	*bracteata*	Bra1	Brazil	–	–	D	D
106884	*macrocephala*	Mac1	Brazil	17°30′S	44°45′W	D	D
93045	*pilosa*	Pil1	Brazil	17°24′S	45°00′W	D	E
94409	*sp.*	Spe1	Brazil	18°14′S	43°36′W	D	F
94410	*sp.*	Spe2	Brazil	18°11′S	43°33′W	D	F
78192	*leiocarpa*	Lei1	Argentina	28°31′S	59°00′W	D	F
92840	*campestris*	Cam1	Brazil	18°35′S	43°26′W	D	G
60692	*grandifolia*	Gra1	Brazil	–	–	D	G
34751	*guianensis*	Gui1	Brazil	21°35′S	48°22′W	D	G
78191	*hippocampoides*	Hip1	Argentina	28°31′S	59°00′W	D	G
11494	*montevidensis*	Mon1	Paraguay	26°25′S	57°08′W	D	G
92843	*tomentosa*	Tom1	Brazil	18°32′S	43°35′W	D	H
73525	*calcicola*	Cal1	Mexico	21°03′N	87°01′W	D	I
91492	*calcicola*	Cal2	Mexico	21°10′N	90°00′W	D	I
49833	aff. *hamata*	Haf1	Brazil	16°15′S	41°32′W	T	AB
110116	aff. *hamata*	Haf2	Venezuela	7°47′N	72°26′W	T	AB
115945	*scabra*	Sca1	Bolivia	16°30′S	63°45′W	T	AB
115952	*scabra*	Sca2	Venezuela	9°39′N	63°54′W	T	AB
55816	aff.*scabra*	scf1	Brazil	13°43′S	40°17′W	T	AB
55871	aff. *scabra*	Scf2	Brazil	15°03′S	40°29′W	T	AB
110175	*sericeiceps*	Ser1	Venezuela	9°47′N	69°35′W	T	AB
100452	*tuberculata*	Tub1	Bahamas	–	–	T	AB?
34148	*sp.* A	Spa1	Honduras	14°01′N	87°01′W	T	AC
76255	*mexicana*	Mex1	Guatemala	14°44′N	90°24′W	T	AC
87484	*mexicana*	Mex2	Mexico	16°52′N	96°45′W	T	AC
33943	*subsericea*	Sub1	Mexico	16°40′N	96°18′W	T	AC
38604	*subsericea*	Sub2	Mexico	16°42′N	92°59′W	T	AC
47477	*sundaica*	Sun1	Indonesia	8°12′S	114°27’E	T	AC
96907	*sundaica*	Sun2	Indonesia	–	–	T	AC
65958	*sympodialis*	Sym1	Ecuador	2°15′S	80°42′W	T	AF
67705	*sympodialis*	Sym2	Ecuador	1°18′S	81°05′W	T	AF
55840	*capitata*	Cap1	Brazil	13°46′S	42°40′W	T	DE
35015	*erecta*	Ere1	Ivory Coast	–	–	H	ABX
69200	*erecta*	Ere2	Nigeria	6°27′N	3°28′E	H	ABX

study phylogenetic relationships among several species (Gillies and Abbott 1996). Results from these studies have improved our understanding of this genus.

The Australian Tropical Forages Genetic Resource Centre (ATFGRC) is a major international centre holding a large collection of *Stylosanthes* species (Schultze-Kraft et al. 1984). An accurate taxonomy for several taxa in this collection is not available due to the difficulties encountered in identifying them on the basis of plant and seed morphology. This paper reports an attempt to identify 6 of these unclassified taxa based on RFLP and STS analyses. In the process, diploid taxa of *Stylosanthes* were classified into basal (genome) groups, and putative genomic structures for several polyploid taxa were identified.

Material and Methods

Genetic stocks

Forty-five genotypes, representing 30 taxa, were used in this study (Table 1). For the selection of these taxa, priority was given to those unclassified and those agronomically important taxa or their putative diploid progenitor species. Wherever possible 2 accessions were selected for each taxon. However, sufficient seed was available for only one accession of several taxa. These included *S. sp.*A, *S. sp.*B, *S. bracteata*, *S. campestris*, *S. hippocampoides*, *S. montevidensis*, *S. sericeiceps*, *S. tomentosa* and *S. tuberculata*. Two accessions of *S. fruticosa* were also selected for this study. However, together with several other accessions, plants of both accessions had perished before DNA could be successfully extracted from them.

DNA extraction

Leaves from two to three mature plants of each genotype were harvested. Freeze-dried leaf tissue was used for DNA extraction following the method described by Liu and Musial (1995).

RFLP and STS analysis

Forty-two single-copy RFLP probes from a *Pst*I library (Liu and Musial 1995) and 14 sets of STS primers (Liu et al. 1996) were used. RFLP and STS procedures were as described in these papers.

Data analysis

RFLP and STS products were scored as the presence (1) or absence (0) of bands. The fraction of bands in common between two genotypes (F) was calculated using the formula of Nei and Li (1979). The McQuitty procedure of SAS based on the unweighted pair group method with arithmetic mean (UPGMA) and the Proc Tree procedure of SAS (SAS Institute 1988) were used to cluster the diploid or tetraploid genotypes on the basis of the dissimilarity (1-F) values. The percentage of DNA fragments of each diploid genotype present in each polyploid genotype was then calculated using a computer program written by Dr. Peter Jones, CSIRO Cunningham Laboratory.

Results and discussion

Ploidy levels of the 45 genotypes

With the use of a single-copy RFLP probe, a diploid genotype often produces a single DNA fragment, a tetraploid two DNA fragments and a hexaploid, three (Fig. 1). Thus, it is possible to putatively infer the ploidy level of a genotype from the RFLP banding patterns of several single-copy probes. RFLP patterns of the 45 genotypes used in this study suggested that 25 were diploids, 18 tetraploids and 2 hexaploids (Table 1).

Apart from the 6 unclassified taxa, ploidy levels for many of the taxa used in this study have been published previously (Stace and Cameron 1984). Chromosome numbers for all but 1 of these published taxa were confirmed by results of this study. The exception was *S. mexicana*. It was previously reported as a diploid based on isozyme patterns (Stace and Cameron 1984). However, the RFLP patterns for both of the accessions used in this study indicated that *S. mexicana* is likely to be an allotetraploid species. Somatic chromosome numbers of these 2 genotypes were counted, and both were found to have 40 chromosomes (data not shown), confirming that both accessions are, indeed, tetraploids.

Basal genomes in *Stylosanthes*

In total, 225 RFLP and STS fragments were scored. Based on these fragments, the 1-F values among the 25 diploid accessions were calculated (Table 2). Using the grouping result of the 5 members of the *S. guianensis* complex as a reference, we arbitrarily classified these diploid genotypes into nine (genome) groups based on their 1-F values. Each of these nine groups was then given a genome symbol, from A to I (Table 1, Fig. 2), to facilitate description of the genomic structures of different taxa in this genus.

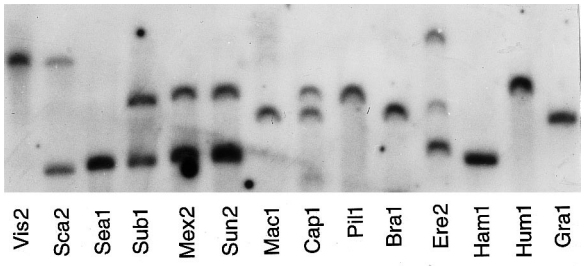

Fig. 1 Autoradiogram derived from probing with SsCS269 to *Dra*I-digested genomic DNA from 14 *Stylosanthes* genotypes (see text for explanation)

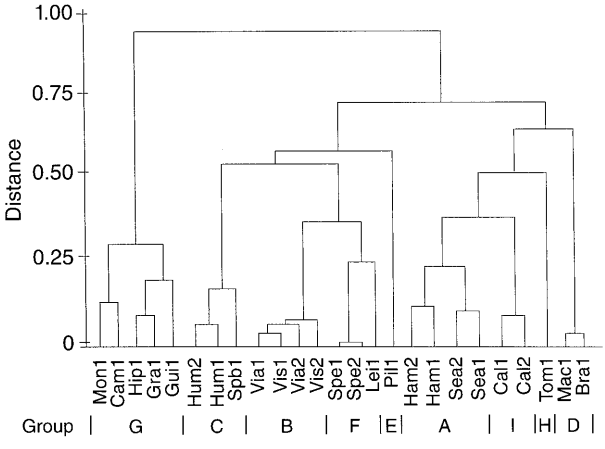

Fig. 2 Phenogram of 25 diploid genotypes of *Stylosanthes* presented as dissimilarity values based on RFLP and STS DNA fragmen

The reasons for using the grouping results of the *S. guianensis* complex as the reference for classifying the diploid taxa are twofold. On the one hand, there can be no doubt that these taxa have evolved from a common basal genome (Williams et al. 1984). On the other hand, the high degree of variation between the members of the *S. guianensis* complex could warrant their separate species status (Kazan et al. 1993b). Thus, it seemed to be reasonable to assign taxa with different genome symbols if the genetic variation between them was higher than that found between members of the *S. guianensis* complex. Furthermore, the existence of intraspecific variations in both the donor and the recipient taxon makes it unlikely to establish a 'perfect match' between them. Rather, similar degrees of matches could be found between genotypes in several taxa. Thus, delimiting more refined groups was not necessarily helpful at this stage.

It was not surprising that some of the groups contained genotypes of different taxa. For example, one group contained the 2 'S. seabrana' and the 2 diploid *S.*

Table 2 Matrix of 1-F values between 25 diploid *Stylosanthes* genotypes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 (Spe1)	0.00	0.00	0.30	0.45	0.48	0.45	0.58	0.62	0.62	0.49	0.45	0.61	0.36	0.36	0.22	0.44	0.54	0.48	0.46	0.53	0.44	0.25	0.26	0.22	0.24
2 (Spe2)		0.00	0.32	0.48	0.48	0.45	0.58	0.63	0.63	0.49	0.46	0.61	0.37	0.37	0.21	0.47	0.52	0.47	0.47	0.52	0.44	0.55	0.27	0.22	0.24
3 (Spb1)			0.00	0.51	0.44	0.48	0.56	0.61	0.63	0.52	0.48	0.59	0.12	0.17	0.37	0.51	0.52	0.51	0.51	0.51	0.46	0.37	0.36	0.37	0.38
4 (Bra1)				0.00	0.49	0.52	0.56	0.62	0.62	0.49	0.46	0.58	0.53	0.51	0.47	0.04	0.57	0.62	0.51	0.52	0.48	0.53	0.52	0.57	0.52
5 (Cal1)					0.00	0.07	0.59	0.06	0.66	0.35	0.28	0.62	0.45	0.44	0.45	0.49	0.56	0.51	0.40	0.38	0.38	0.51	0.51	0.54	0.52
6 (Cal2)						0.00	0.62	0.69	0.67	0.27	0.25	0.62	0.47	0.45	0.43	0.52	0.58	0.49	0.30	0.27	0.38	0.48	0.49	0.51	0.51
7 (Cam1)							0.00	0.25	0.31	0.56	0.57	0.26	0.61	0.61	0.58	0.58	0.13	0.51	0.58	0.58	0.50	0.52	0.52	0.58	0.55
8 (Gra1)								0.00	0.13	0.66	0.71	0.09	0.67	0.70	0.60	0.62	0.22	0.58	0.67	0.69	0.58	0.60	0.60	0.64	0.61
9 (Gui1)									0.00	0.66	0.72	0.21	0.68	0.70	0.62	0.62	0.28	0.58	0.67	0.70	0.55	0.60	0.59	0.64	0.61
10 (Ham1)										0.00	0.12	0.58	0.56	0.49	0.47	0.48	0.54	0.54	0.15	0.17	0.50	0.50	0.51	0.53	0.46
11 (Ham 2)											0.00	0.65	0.50	0.49	0.47	0.46	0.51	0.58	0.22	0.24	0.44	0.48	0.47	0.51	0.46
12 (Hip1)												0.00	0.66	0.64	0.58	0.60	0.19	0.58	0.62	0.67	0.58	0.55	0.56	0.61	0.60
13 (Hum1)													0.00	0.05	0.44	0.53	0.57	0.59	0.55	0.55	0.51	0.47	0.48	0.48	0.46
14 (Hum2)														0.00	0.41	0.51	0.56	0.57	0.49	0.48	0.44	0.49	0.49	0.44	0.49
15 (Lei1)															0.00	0.49	0.59	0.54	0.46	0.53	0.48	0.38	0.35	0.37	0.32
16 (Mac1)																0.00	0.59	0.61	0.50	0.52	0.50	0.51	0.49	0.55	0.50
17 (Mon1)																	0.00	0.61	0.60	0.64	0.54	0.52	0.50	0.54	0.56
18 (Pil1)																		0.00	0.55	0.56	0.57	0.43	0.47	0.47	0.48
19 (Sea2)																			0.00	0.09	0.47	0.46	0.47	0.48	0.50
20 (Sea1)																				0.00	0.49	0.51	0.53	0.54	0.51
21 (Tom1)																					0.00	0.43	0.47	0.46	0.48
22 (Vis1)																						0.00	0.05	0.04	0.07
23 (Vis2)																							0.00	0.08	0.08
24 (Via1)																								0.00	0.06
25 (Via2)																									0.00

hamata genotypes (Fig. 2). 'S. seabrana' was recognised (although not as yet formally described) as a separate species recently by B. Maass and L. 't Mannetje (personal communication) based on its morphological, cytogenetical (Liu and Musial 1997) and *Rhizobium* affinity (Date *et al.* 1996). The average dissimilarity value between 'S. seabrana' and *S. hamata* (22.9%) was significantly higher than the intraspecific variation generally found in *Stylosanthes*, but it was at the lower end of the spectrum of interspecific variations (Kazan et al. 1993a). Considering the facts that they are reasonably distinct and that they are widely separated geographically (Williams et al. 1984; Date et al. 1996), two different genome symbols, A1 and A2, were assigned to 'S. seabrana' and diploid *S. hamata*, respectively.

The high percentage of DNA fragments shared between the single accession of *S. bracteata* and that of *S. macrocephala* was surprising. The 1-F between them (4.3%) was well below the average found between genotypes of the same taxon (6.8% in this study). Clearly, it is unlikely that these two accessions belong to 2 different species. Similarly, genetic variation between the 2 *S.* aff. *viscosa* and the 2 *S. viscosa* accessions (8.0%) indicated that they are not sufficiently divergent to be treated as different species.

The 2 genotypes representing the taxon *S. sp*. seemed to be distinct from any other species used in this study. However, designation of a new species to this taxon would have to wait until its similarity with all known *Stylosanthes* species is determined. A similar situation may also exist for *S. sp*.B.

The reason for assigning the symbol A to the genome of the group containing 'S. seabrana' and *S. hamata* was that they were the constituent genomes for the 2 most widely used allotetraploid species, *S. scabra* and *S. hamata* (Cameron *et al.* 1996). For the same reason, the symbol B was given to the genome of the group containing *S. viscosa*, which contributed the second constituent genome of *S. scabra* (Liu and Musial 1997), and C was given to the group containing *S. humilis*, the second genome of tetraploid *S. hamata* (Curtis et al. 1995).

Putative diploid donors for polyploid taxa

A likely candidate for a donor species is one that not only shares the majority of its DNA fragments with the recipient but also has a natural distribution that overlaps with that of the recipient. Unfortunately, due to the lack of taxonomic stability, information on geographical distributions for many *Stylosanthes* species is incomplete (Williams et al. 1984).

Another factor that could affect the identification of a donor species is the existence of intraspecific variation. As mentioned before, the variation makes a 'perfect match' between donor and recipient very unlikely. High levels of intraspecific variation also make 'boundaries' between taxa less clear. The greater the extent of this variation, the more difficult it would be for the unambiguous identification of donor-recipient relationships. As a result, more than 1 diploid genotype could share a similar percentage of DNA fragments with 1 polyploid genotype. Similarly, 2 different genotypes of the same taxon could share markedly different percentages of fragments with a third genotype. For these reasons, we have emphasised the best matches between diploid and polyploid taxa, while realising that further research may identify closer relationships.

Based on the percentages of diploid DNA fragments present in polyploid genotypes (Table 3) and the dissimilarity values among the tetraploid genotypes (Table 4), the 20 polyploid genotypes were grouped into five classes.

Class I contains the single tetraploid *S. capitata* genotype only. More than 80% of its DNA fragments had counterparts in either the diploid *S. pilosa* or the diploid *S. bracteata/S. macrocephala* genotypes, while none of the other diploids shared a high percentage of fragments with it (Table 3). Thus, the likely genomic structure for *S. capitata* should be DDEE. An example of this additive banding pattern between these 4 taxa can be seen in Fig. 1.

The relationship between *S. capitata* and its putative diploid donor species was supported by their overlapping geographical distributions. All 4 taxa occur in the same region in Brazil (Williams et al. 1984). *S. capitata* also occurs in eastern Venezuela but not with any of the other 3 diploid taxa. It seems likely that genotypes of *S. capitata* evolved in Brazil and then dispersed to eastern Venezuela. It is also interesting to note that *S. capitata* was apparently the first example of a tetraploid be formed between diploid species belonging to one of the two sections of *Stylosanthes* (Stace and Cameron 1984).

Both *S. capitata* (Genome DE) and *S. macrocephala* (Genome D) have shown agronomic potential in Brazil, with the latter being found to be highly resistant to anthracnose. However, as demonstrated by Sousa Costa and Ferreira (1984), interspecific hybrids between these 2 species are very unlikely as they differ in ploidy levels. Rather, the production of synthetic populations by mixing resistant *S. macrocephala* accessions with productive *S. capitata* accessions has been the main breeding strategy (Grof et al. 1997). Results from this study showed that it should be possible to transfer genes from *S. macrocephala* to *S. capitata*. This can be achieved by crossing *S. macrocephala* (Genome D) and *S. pilosa* (Genome E) to synthesise allotetraploids and then hybridizing these artificial allotetraploids with those natural *S. capitata* genotypes.

Class II contains genotypes of *S. scabra*, *S.* aff. *scabra*, *S. sericeiceps*, *S.* aff. *hamata* and *S. tuberculata*. Genotypes of the first 4 tetraploid taxa shared high percentages of DNA fragments with diploid genotypes in both Groups A and B (Table 3). The average 1 – F value (8.4%) among genotypes of these four taxa (Table 4) also supports the notion that they have a similar genomic structure.

Table 3 Percentaes of DNA fragments of diploids present in polyploids

Genome	Diploid	Polyploid																			
		Spa1	Cap1	Ere1	Ere2	Haf1	Haf2	Mex1	Mex2	Sca1	Sca2	Scf1	Scf2	Ser1	Sub1	Sub3	Sun1	Sun2	Sym1	Sym2	Tub1
A	Sea1	0.67	0.50	0.81	0.88	0.67	0.79	0.75	0.71	0.92	0.67	0.81	0.69	0.83	0.75	0.67	0.54	0.69	0.71	0.65	0.77
A	Sea2	0.70	0.57	0.85	0.91	0.67	0.87	0.83	0.74	0.91	0.67	0.89	0.72	0.83	0.80	0.72	0.61	0.74	0.76	0.67	0.80
A	Ham1	0.79	0.57	0.79	0.88	0.67	0.86	0.90	0.81	0.90	0.67	0.88	0.69	0.79	0.90	0.83	0.67	0.83	0.76	0.69	0.79
A	Ham2	0.81	0.49	0.87	0.70	0.74	0.70	0.79	0.85	0.79	0.68	0.70	0.72	0.70	0.77	0.85	0.51	0.85	0.66	0.70	0.66
B	Vis1	0.58	0.56	0.82	0.82	0.82	0.80	0.56	0.60	0.78	0.80	0.80	0.69	0.76	0.58	0.60	0.42	0.62	0.51	0.62	0.73
B	Vis2	0.60	0.58	0.86	0.81	0.84	0.79	0.58	0.63	0.79	0.81	0.81	0.70	0.77	0.58	0.63	0.47	0.65	0.56	0.65	0.77
B	Via1	0.62	0.51	0.79	0.79	0.82	0.74	0.56	0.62	0.74	0.77	0.74	0.64	0.74	0.59	0.62	0.49	0.64	0.56	0.67	0.72
B	Via2	0.58	0.53	0.83	0.78	0.83	0.75	0.55	0.58	0.83	0.80	0.75	0.73	0.83	0.55	0.63	0.45	0.63	0.60	0.68	0.75
C	Spb1	0.82	0.47	0.67	0.69	0.64	0.60	0.76	0.82	0.76	0.62	0.60	0.53	0.58	0.80	0.84	0.62	0.87	0.62	0.71	0.06
C	Hum1	0.80	0.50	0.55	0.66	0.55	0.52	0.82	0.80	0.72	0.52	0.52	0.48	0.55	0.89	0.80	0.68	0.82	0.57	0.61	0.55
C	Hum2	0.87	0.43	0.59	0.61	0.54	0.48	0.72	0.85	0.67	0.50	0.48	0.46	0.48	0.78	0.85	0.61	0.89	0.57	0.61	0.50
D	Bra1	0.37	0.80	0.46	0.51	0.39	0.49	0.46	0.39	0.53	0.37	0.47	0.41	0.47	0.46	0.41	0.34	0.42	0.42	0.44	0.46
D	Mac1	0.39	0.81	0.49	0.51	0.42	0.53	0.47	0.40	0.56	0.40	0.51	0.44	0.49	0.47	0.42	0.35	0.44	0.42	0.44	0.49
E	Pil1	0.40	0.83	0.50	0.52	0.48	0.52	0.46	0.46	0.56	0.46	0.52	0.42	0.52	0.48	0.42	0.37	0.44	0.44	0.50	0.50
F	Spe1	0.68	0.60	0.83	0.78	0.80	0.73	0.65	0.73	0.73	0.73	0.73	0.70	0.75	0.63	0.73	0.53	0.75	0.70	0.83	0.73
F	Spe2	0.69	0.59	0.82	0.79	0.79	0.74	0.64	0.72	0.72	0.74	0.74	0.69	0.74	0.64	0.72	0.54	0.74	0.69	0.82	0.74
F	Lei1	0.63	0.56	0.76	0.68	0.66	0.61	0.59	0.63	0.66	0.63	0.63	0.63	0.66	0.56	0.68	0.49	0.68	0.66	0.83	0.63
G	Cam1	0.43	0.57	0.50	0.55	0.45	0.52	0.45	0.48	0.55	0.45	0.55	0.43	0.50	0.48	0.43	0.36	0.43	0.38	0.43	0.50
G	Gra1	0.33	0.41	0.43	0.43	0.41	0.43	0.33	0.33	0.39	0.41	0.45	0.37	0.39	0.33	0.35	0.29	0.35	0.31	0.37	0.41
G	Gui1	0.35	0.44	0.47	0.47	0.44	0.47	0.35	0.35	0.42	0.44	0.49	0.04	0.42	0.35	0.37	0.30	0.37	0.30	0.37	0.44
G	Hip1	0.35	0.45	0.43	0.53	0.37	0.49	0.39	0.37	0.47	0.41	0.51	0.39	0.47	0.39	0.37	0.33	0.37	0.37	0.41	0.47
G	Mon1	0.41	0.45	0.45	0.50	0.39	0.48	0.39	0.41	0.48	0.43	0.50	0.41	0.48	0.41	0.41	0.39	0.41	0.36	0.41	0.48
H	Tom1	0.56	0.54	0.63	0.71	0.54	0.54	0.56	0.56	0.61	0.51	0.56	0.46	0.56	0.59	0.54	0.37	0.56	0.46	0.51	0.54
I	Cal1	0.77	0.66	0.71	0.77	0.63	0.69	0.77	0.71	0.77	0.66	0.74	0.60	0.66	0.80	0.71	0.51	0.74	0.69	0.71	0.69
I	Cal2	0.77	0.52	0.75	0.73	0.66	0.66	0.70	0.75	0.68	0.66	0.68	0.61	0.61	0.73	0.75	0.48	0.77	0.64	0.66	0.64

Table 4 Matrix of 1-F values between 18 tetraploid *Stylosanthes* genotypes

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	Haf1 (AB)	0.00	0.15	0.07	0.08	0.03	0.02	0.12	0.10	0.37	0.32	0.34	0.35	0.34	0.33	0.33	0.31	0.28	0.44
2	Haf2 (AB)		0.00	0.14	0.10	0.13	0.13	0.06	0.09	0.32	0.31	0.36	0.31	0.34	0.36	0.35	0.21	0.33	0.46
3	Sca1 (AB)			0.00	0.09	0.04	0.07	0.10	0.11	0.36	0.28	0.33	0.32	0.33	0.38	0.33	0.32	0.28	0.42
4	Sca2 (AB)				0.00	0.06	0.06	0.07	0.06	0.38	0.35	0.39	0.38	0.38	0.40	0.47	0.34	0.33	0.47
5	Scf1 (AB)					0.00	0.01	0.12	0.10	0.35	0.31	0.33	0.35	0.33	0.35	0.32	0.31	0.27	0.44
6	Scf2 (AB)						0.00	0.11	0.08	0.35	0.31	0.36	0.33	0.34	0.34	0.34	0.31	0.31	0.43
7	Ser1 (AB)							0.00	0.06	0.35	0.33	0.35	0.35	0.35	0.35	0.35	0.28	0.29	0.45
8	Tub1 (AB)								0.00	0.36	0.32	0.34	0.36	0.34	0.36	0.33	0.29	0.27	0.43
9	Spa1 (AC)									0.00	0.04	0.04	0.02	0.03	0.05	0.02	0.20	0.29	0.57
10	Mex1 (AC)										0.00	0.04	0.02	0.03	0.10	0.02	0.23	0.25	0.51
11	Mex2 (AC)											0.00	0.03	0.04	0.07	0.03	0.21	0.30	0.52
12	Sub1 (AC)												0.00	0.02	0.08	0.02	0.21	0.26	0.54
13	Sub2 (AC)													0.00	0.05	0.01	0.22	0.28	0.53
14	Sun1(AC)														0.00	0.06	0.23	0.31	0.55
15	Sun2 (AC)															0.00	0.21	0.24	0.52
16	Sym1 (AF)																0.00	0.16	0.51
17	Sym2 (AF)																	0.00	0.43
18	Cap1 (DE)																		0.00

The single genotype representing *S. tuberculata* shared a high percentage (up to 80%) of DNA fragments with diploid genotypes in Group A, but its second genome shared only moderate percentages of its fragments with those of the diploids in Group B (Table 3). However, the average 1-F value between this *S. tuberculata* genotype and genotypes of *S. scabra*, *S.* aff. *scabra*, *S. sericeiceps* and *S.* aff. *hamata* (8.6%, Table 4) clearly showed that these taxa have a similar genomic structure. The result that *S. tuberculata* is likely to share the same genome with *S. scabra* is in agreement with Mannetje (1984), who postulated that the former formed part of the latter.

The 5 members of this class have a geographical range from Brazil through Columbia and Venezuela to the West Indies. Of these, *S. scabra* has the broadest range, which not only overlaps or is contiguous with the other 4 members (Williams *et al*. 1984), but also encompasses the geographical range of the putative donor species, *S. viscosa* and *S. hamata*/'S. seabrana'.

Class III contains the 2 accessions belonging to *S. sympodialis*. One of their genomes shared high percentages (more than 80%) of fragments with each of the 3 diploid members in Group F, and its other genome shared the highest percentage of fragments with members in Group A (Table 3).

Williams et al. (1984) maintained that the occurrence of *S. sympodialis* on neutral to slightly alkaline soils as well as its distinct distribution from *S. scabra*, which occurs on acid soils, should warrant different species status for the 2 taxa. This was supported by findings in this study that *S. sympodialis* has a genome structure of AAFF, whereas *S. scabra* and *S. sericeiceps* share a genome structure of AABB. However, it is of note that genomes B and F are closely related among all of the diploid taxa used in this study (Table 2; Fig. 2), and that the differences among them is similar to the variation found between members of the *S. guianensis* complex.

Class IV contains genotypes of *S. mexicana, S. subsericea, S. sundaica* and *S. sp*.A. Similar to the allotetraploid *S. hamata* (Curtis et al. 1995), these 4 taxa all seem to have a genome structure of AACC. Accessions of these 4 taxa shared high percentages of DNA fragments with diploid genotypes in both Groups A and C (Table 3). Genotypes of these 4 taxa often produced the same restriction pattern. The 1-F value among these 4 taxa (3.9%; Table 4) also suggested that, at DNA level, they are not sufficiently divergent to be treated as different species.

It was not surprising that *S. mexicana* and *S. subsericea* share a similar genome structure. The geographical distributions of these 2 species overlap in Mexico and Guatemala (Williams et al. 1984), and there are no clear taxonomic differences between them (Mannetje 1984). There is no record of a direct comparison between *S. sundaica* and other polyploid species, however, morphologically both *S. sundaica* and *S. subsericea* are similar to *S. humilis* (Burt 1984). *S. sundaica* and *S. humilis* are so similar that Mohlenbrock (1958) and Pedley (1977) once considered them to be the same species.

Class V contains only the 2 accessions of *S. erecta*. Two of its three genomes shared high percentages of DNA fragments with diploids in Group A (up to 91%) and Group B/Group F (up to 86%) respectively (Table 3). However, in the third genome of this species approximately 30% of its fragments were not found in any of the diploids used in this study. This lack of clear relationships could, again, be due to intraspecific variation in both the diploid donor and the polyploid recipient species or be due to the simple fact that the third diploid donor species was not included in this study. It is of note that *S. erecta* is 1 of 3 *Stylosanthes* species endemic to Africa, with the other 2 being *S. fruticosa* and *S. orbiculata.* A study of the latter 2 species might help to clarify the evolution of *S. erecta*.

Single genes, such as *Ph1* in *Triticum* (Riley and Chapman 1958), could have a dramatic effect on chro-

mosome pairing and thus on gene transfers. However, the overall similarity between two genotypes is a good indicator of how likely it is that they would cross and produce fertile progeny. Several diploid groups and tetraploid classes identified in this study contain genotypes from more than one taxon. It would be highly desirable to investigate if genotypes from these different taxa within the same groups or classes can hybridize and produce fertile progeny. Such work would not only be helpful in clarifying their species status but would also be critical for designing strategies in maximizing the utilisation of genetic variations in a breeding programme.

Acknowledgements We are very grateful to Don Cameron, Dick Date, Bryan Hacker and Len 't Mannetje for their constructive suggestions on this study, Peter Jones and David Eagle for help with statistical analysis, and Ron Williams for selection of plant material. The experiments reported in this paper comply with the current laws of Australia in which the experiments were performed.

References

Burt RL (1984) Natural variation in *Stylosanthes*. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, London New York, pp 103–123

Cameron DF (1967) Chromosome number and morphology of some introduced *Stylosanthes* species. Aust J Agric Res 18:375–379

Cameron DF, Edye LA, Chakraborty S, Manners JM, Liu CJ, Date RA, Boland RM (1996) An integrated program to improve anthracnose resistance in *Stylosanthes* – a review. In: Asghar M (ed) Proc 8th Australian Agronomy Conf, Toowoomba. The Australian Institute of Agricultural Science, Carlton, Victoria, Australia, pp 112–115

Curtis MD, Cameron DF, Manners JM (1995) Molecular evidence that diploid *Stylosanthes humilis* and diploid *Stylosanthes hamata* are progenitors of allotetraploid *Stylosanthes hamata* cv 'Verano'. Genome 38:344–348

Date RA, Edye LA, Liu CJ (1996) *Stylosanthes* sp. aff. *Scabra*: a potential new forage plant for northern Australia. Trop Grassl 30:131

Edye LA, Cameron DF (1984) Prospects for *Stylosanthes* improvement and utilization. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, London New York, pp 571–587

Gillies ACM, Abbott RJ (1996) Phylogenetic relationships in the genus *Stylosanthes* (Leguminosae) based upon chloroplast DNA variation. Plant Syst Evol 200:193–211

Glover BJ, Gillies ACM, Abbott RJ (1994) Use of the polymerase chain reaction to investigate the delimitation of two agronomically important species of *Stylosanthes* (Aubl.) Sw. Bot J Scot 47:83–96

Grof B, Fernandes CD, Almeida CB, dos Santos AV (1997) Development of a multicross cultivar of *Stylosanthes* spp. In: Proc 18th Int Grassl Congr. Winnipeg/Saskatoon, Canada, pp 4:31–4:32

Kazan K, Manners JM, Cameron DF (1993a) Genetic variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers. Theor Appl Genet 85:882–888

Kazan K, Manners JM, Cameron DF (1993b) Genetic relationships and variation in the *Stylosanthes guianensis* (Aubl.) Sw. species complex assessed by random amplified polymorphic DNA. Genome 36:43–49

Liu CJ, Musial JM (1995) Restriction fragment length polymorphism detected by cDNA and genomic DNA clones in *Stylosanthes*. Theor Appl Genet 91:1210–1213

Liu CJ, Musial JM (1997) *Stylosanthes* sp. aff. *S. scabra*: a putative diploid progenitor of *Stylosanthes scabra*. Pl Syst Evol 208:99–105

Liu CJ, Musial JM, Smith FW (1996) Evidence for a low level of genomic specificity of sequence-tagged-sites in *Stylosanthes*. Theor Appl Genet 93:864–868

Mannetje L't (1984) Considerations on the taxonomy of the genus *Stylosanthes*. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, London New York, pp 1–21

Mohlenbrock RH (1958) A revision of the genus *Stylosanthes*. Ann Mo Bot Garden 44:299–355

Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Pedley L (1977) Notes on Leguminosae I. Austrobaileya 1:25–42

Riley R, Chapman V (1958) Genetic control of the cytologically diploid behaviour of hexaploid wheat. Nature 182:713–758

SAS Institute (1988) SAS language guide for personal computers. Release 6.03 edn. SAS Institute, Cary, N.C.

Schultze-Kraft R, Reid R, Williams RJ, Coradin L (1984) The existing *Stylosanthes* collections. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, London New York, pp 125–146

Sousa Costa NM, Ferreira MB (1984) Some Brazilian species of *Stylosanthes*. In: Stace HM., Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, London New York, pp 23–48

Stace HM, Cameron DF (1984) Cytogenetics and evolution of *Stylosanthes*. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, London New York, pp 49–72

Williams RJ, Reid R, Schultze-Kraft R, Sousa Costa NM, Thomas BD (1984) Natural distribution of *Stylosanthes* In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, London New York, pp 73–101